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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 15 (2005) 2311–2314

A new development of matrix metalloproteinase inhibitors: twin hydroxamic acids as potent inhibitors of MMPs

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Received 25 November 2004; revised 24 February 2005; accepted 2 March 2005
Available online 30 March 2005

Abstract—Starting from the observation that the $CbzNH(CH_2)_2$ side chain of the potent MMP-2/MMP-14 inhibitor, benzyl-(3*R*)-4-(hydroxyamino)-3-[isopropoxy(1,1′-biphenyl-4yl-sulfonyl)amino]-4-oxobutylcarbamate, (*R*)-**1** lies in a hydrophobic region (S1) exposed to the solvent of the protease active site, we hypothesized that an aminoethylcarboxamido chain structurally related to that of (*R*)-**1** might be an useful tool to bind another linker stretching out from the protein. This would be able to interact either with a enzyme region adjacent to the active site, or with other molecules of matrix metalloproteinases (MMPs), or other proteins of the extracellular matrix (ECM) that may be involved in the enzyme activation. On these basis we describe new dimeric compounds of type **2**, twin hydroxamic acids, obtained by the joint of two drug entities of (*R*)-**1** linked in P1 by extendable semirigid linkers. Type **2** compounds are potentially able to undergo more complex inhibitor–enzyme interactions than those occurring with monomeric compounds of type **1**, thus influencing positively the potency, selectivity and/or cytotoxicity of the new compounds.

During tumour progression, an overexpression of matrix metalloproteinases (MMPs) is often responsible for the deregulation of extracellular matrix (ECM) functions.[1,2] Normally, the homeostasis of MMPs is maintained by tissue inhibitors (TIMPs), but in cancer progression, control over MMPs activity is lost.[3] Other proteases, such as uPA, MMPs and furin-like serine proteases, are responsible for the activation of specific MMPs, such as MMP-2 (gelatinase A), MMP-9 (gelatinase B) and MMP-14 (a membrane-associated MMP, which is the principal activator of pro-MMP-2). These three specific MMPs play a significant role in some neoplastic processes, and are directly involved in metastatic tumour dispersion and angiogenesis.[4–8] More recently, MMP-2 has been shown to play other important roles in tumours, for example, increasing the resistance to apoptosis, activating EGF receptors and promoting cellular proliferation.[9–12] All these data, taken together, indicate that MMP-2, MMP-9 and MMP-14 may represent important targets to develop new potential anticancer drugs.[13–21] As a matter of fact, the recent development of some synthetic MMPi (matrix metalloproteinase inhibitors) possessing a good potency and selectivity towards the two gelatinases A and B, together with the discovery that some of these inhibitors that are active on MMP-2 exhibit important pro-apoptotic effects on tumour cell cultures, has confirmed the possibility of their potential use as anti-tumour agents.[22–29] Nowadays, new compounds possessing inhibitory activity and selectivity on the MMPs, which are over-expressed in some kinds of tumours, are viewed as useful tools in particular with a view to the control of the viability and invasiveness of cancer cells.[2,15]

In previous studies on new MMPi, we developed a class of arylsulfonamido-based hydroxamic acid inhibitors of type **A**, substituted on their sulfonamido nitrogen with an oxyalkyl side chain, instead of the hydrogen atom of type **B** compounds, or an alkyl side chain of type **C** compounds. This simple structural modification allowed us to obtain new potent and selective inhibitors of MMP-2 and MMP-9, which are able to block in vitro

Keywords: MMP-inhibitors; MMP-2/MT1-MMP selective inhibitors; Antiangiogenic agents; Twin hydroxamic acids; MMPi; Arylsulfonamido-based hydroxamate.
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doi:10.1016/j.bmcl.2005.03.002

tumour cell invasion.[30] Among the two type **A** inhibitors, the ones with the (*R*) configuration, which bear on the carbon alpha to the hydroxamic group an alkyl group (R) of increasing hindrance, showed better biopharmacological properties with respect to the unsubstituted analogues. In particular, the compound of type **A** in which R = $(CH_2)_2NHCbz$ R_1 = *i*-Pr and R_2 = Ph ((*R*)-**1**) showed a very good MMP inhibitory profile, with a high potency towards MMP-2 (IC_{50} = 0.41 nM), MMP-9 (IC_{50} = 16 nM) and MMP-14 (IC_{50} = 7.7 nM), and was able to block angiogenesis in the chemioinvasion model on HUVEC cells completely, at a submicromolar concentration.[31] A docking study carried out on the complex of (*R*)-**1** with MMP-2 showed that its $CbzNH(CH_2)_2$ side chain lies in a hydrophobic region of the active site exposed to the solvent (S1) (see green arrow in Fig. 1), without hindering the fit of the inhibitor with the enzyme.[32]

This observation prompted us to hypothesize that an aminoethylcarboxamido chain structurally related to that of (*R*)-**1** might be a useful tool to bind another linker stretching out from the protein, able to interact either with an enzyme region adjacent to the active site, or with other MMPs molecules or other proteins of the ECM, which may be involved in the enzyme activation. We believe that the possibility, for the dimeric compounds of type **2**, to give an inhibitor–enzyme interaction more complex than that allowed for monomeric compounds of type **1**, may influence positively the potency and/or selectivity of the new compounds, compared with those of **1**. As a result, we designed the new twin inhibitors (*R*,*R*)-**2a**,**b**, formally obtained by linking two of the fundamental frames (*R*)-**3**[31] of (*R*)-**1** by a spacer like an isophthaloyl group [(*R*,*R*)-**2a**] or an *N*,*N*′-bis(4-aminobutanoyl)-isophthalamide moiety [(*R*,*R*)-**2b**], in order to study the effects of this type of structural manipulation on the MMP-2/MMP-9/MMP-14 inhibitory activity of compounds of type **1**. As a core, in the linker in this study, we used the isophthaloyl group, either simple or symmetrically linked to two γ-aminobutyric portions, in view of its ability to confer a partial rigidity to the linker chain and potentially to interact with appropriate parts of the enzyme (Fig. 2).[32]

There are numerous examples, in the field of drug research, of twin drugs containing two pharmacophoric groups linked in a single molecule, some of the hydroxamic acid type, but none in the field of MMPi.[33–36]

The synthesis of the two twin inhibitors (*R*,*R*)-**2a** and (*R*,*R*)-**2b** is outlined in Scheme 1. As a start, the Cbz derivative (*R*)-**4** was hydrogenated with H_2 and Pd/10% on charcoal in a 1:1 MeOH/AcOH mixture to yield the deprotected salt (*R*)-**5**.[31] Acylation of (*R*)-**3** with the appropriate acyl dichloride (**6a**) or (**6b**), in anhydrous DMF in the presence of *N*-methylmorpholine (NMM), gave the dimeric *tert*-butyl esters (*R*,*R*)-**7a** and (*R*,*R*)-**7b**, respectively. Acidic cleavage of **7a** and **7b** to acids (*R*,*R*)-**8a** and (*R*,*R*)-**8b**, followed by their reaction with *O*-(*tert*-butyldimethylsilyl)hydroxylamine (TBDMSO–NH_2) in the presence of EDCI, gave the *O*-silylate bishydroxamate (*R*,*R*)-**9a** and (*R*,*R*)-**9b**. Acid cleavage of (*R*,*R*)-**9a**,**b** yielded the corresponding bishydroxamic acids (*R*,*R*)-**2a** and (*R*,*R*)-**2b**. The acid dichloride **6b**, used for the synthesis of (*R*,*R*)-**7b**, was prepared by acylation of 4-aminobutyric acid **10** with isophthaloyl dichloride **6a**, to yield the symmetrical diacid **11b**, which was then converted to **6b** with oxalyldichloride in a pyridine/toluene mixture.

The bishydroxamates (*R*,*R*)-**2a** and (*R*,*R*)-**2b**, the dimeric acid (*R*,*R*)-**8a**, a precursor of (*R*,*R*)-**2a**, and the monomeric reference inhibitors (*R*)-**1**, whose molecular skeleton is duplicated in the twin inhibitor (*R*,*R*)-**2a**,**b**,

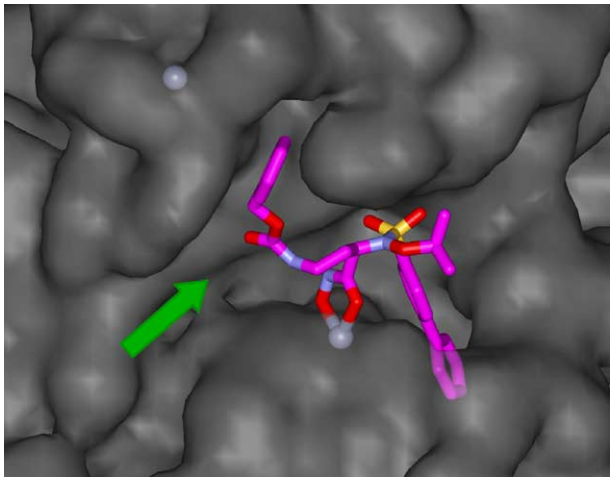


Figure 1. Docking of MMP-2 with monomeric inhibitor (*R*)-**1**.

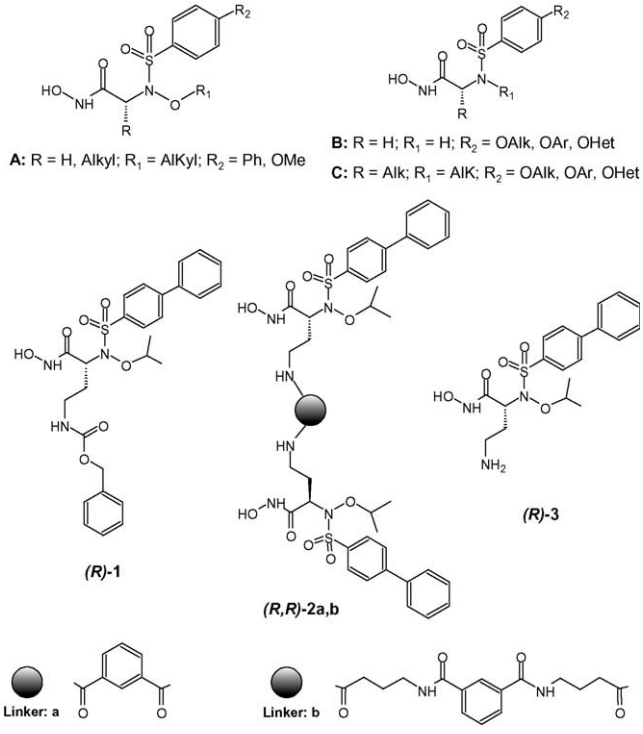
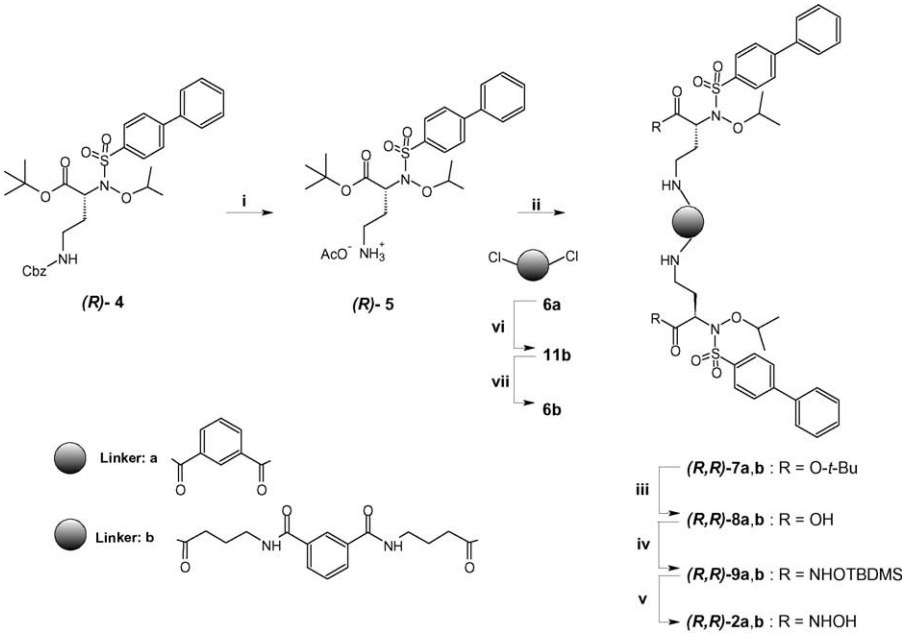


Figure 2.



Scheme 1. Reagents and conditions: (i): H_2, Pd/C 10%, MeOH, AcOH; (ii) **6a** or **6b**, NMM, anhyd DMF; (iii) TFA, anhyd DCM; (iv) $TBDMSONH_2$, EDCI, anhyd DCM; (v) TFA, anhyd DCM; (vi) $H_2N(CH_2)_3CO_2H$, (**10**) NaOH, Et_2O, H_2O; (vii) $C_2O_2Cl_2$, Py, toluene.

were tested on some MMPs (1, 2, 9 and 14) to evaluate their inhibitory properties.[30]

The results, expressed in terms of their IC_{50} and selectivity for MMP-2, are reported in Table 1.

For MMP-2, the two dimers (*R*,*R*)-**2a** and (*R*,*R*)-**2b** show an IC_{50} in the nM range, which is 480- and 24-fold more than that of the monomeric inhibitor (*R*)-**1**, respectively. In the case of MMP-1, only (*R*,*R*)-**2b** shows an appreciable activity, with a submicromolar IC_{50} value, which is 40-fold higher than that displayed towards MMP-2, whilst (*R*,*R*)-**2a** and (*R*)-**1** are practically inactive.

The two dimers (*R*,*R*)-**2a** and (*R*,*R*)-**2b** show an IC_{50} value in the submicromolar range against MMP-9, but in this case dimer (*R*,*R*)-**2b** is twice as potent as (*R*)-**1**, while (*R*,*R*)-**2a** proves to be less potent. Both (*R*,*R*)-**2a** and (*R*,*R*)-**2b** show an MMP-9/MMP-2 selectivity lower than (*R*)-**1**. As regards MMP-14, the twin inhibitor (*R*,*R*)-**2b** shows a high inhibitory potency, albeit with an IC_{50} value four times higher than that of the reference compound (*R*)-**1**; on the contrary, compound (*R*,*R*)-**2a** shows an IC_{50} value in the micromolar range. Both (*R*,*R*)-**2a** and (*R*,*R*)-**2b** are less selective than (*R*)-**1** on the MMP-2 enzyme.

As expected for a compound lacking the hydroxamic acid moiety, the carboxylic analogue of (*R*,*R*)-**2a** ((*R*,*R*)-**8a**) proved to be practically inactive towards the two MMPs screened, MMP-2 and MMP-9.

The more active of the two twin inhibitors ((*R*,*R*)-**2b**) was evaluated for cytotoxicity by means of the Trypan Blue assay on HT1080 cells, in comparison with the monomeric analogue (*R*)-**1** (see Table 2).[37]

As can be seen, (*R*,*R*)-**2b** is well tolerated by the cells at a dose lower than 1 μM; cytotoxic effects for (*R*,*R*)-**2b** become significant at a dose of 10 μM, where this compound shows 10% dead cells. At this same dose, the monomeric analogue (*R*)-**1** is about four times more toxic, with 35% dead cells.

These preliminary results indicate that, even if the dimerization of MMP inhibitors of type **A**, like (*R*)-**1**, appears to be unable to enhance the selectivity of the new compounds towards the MMP screened, nevertheless, at least in the case of (*R*,*R*)-**2b**, it appears to

Table 1. MMPi profile of (*R*,*R*)-**2a**,**b**, **-8a** dimers and monomeric inhibitor (*R*)-**1**

Compound	IC_{50} nM (±SD)[a]			
	MMP-1	MMP-2	MMP-9	MMP-14
(*R*,*R*)-**2a**	10,000 ± 1333 (50)[b]	197 ± 42	983 ± 40 (4.9)[b]	1490 ± 130 (7.6)[b]
(*R*,*R*)-**2b**	397 ± 79 (40)[b]	9.8 ± 2	8.3 ± 1.4 (0.8)[b]	34 ± 14 (3.5)[b]
(*R*,*R*)-**8a**	n.t.	>1000	>30,000	n.t.
(*R*)-**1**	>3000 (>7300)[b]	0.41 ± 0.01	16 ± 2 (39)[b]	7.7 ± 2 (18.7)[b]

[a] MMPi assay.[30]

[b] Selectivity for MMP-2 over each of the other MMPs is expressed as the ratio of the IC_{50} value for MMPn over the value for MMP-2.

Table 2. Cytotoxicity[a] with twin inhibitor (*R*,*R*)-**2b** and monomeric inhibitor (*R*)-**1**

Compound	% Dead cells[b]		
	1 μM	5 μM	10 μM
(*R*,*R*)-**2b**	0	5	10
(*R*)-**1**	4	20	35

[a] Trypan Blue test on HT1080 cells.
[b] Dead cells over total cells, living and dead, percentage at dose screened.[37]

improve the activity on MMP-1 and MMP-9. Moreover, dimerization of (*R*)-**1** to (*R*,*R*)-**2b** seems to reduce the cellular cytotoxicity of this new class of twin inhibitors appreciably.

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37. HT1080 cells stably transfected to overexpress MT1 MMP were seeded into 24-well culture dishes at 10^5 cells/well in selection medium and incubated at 37 °C overnight. The following day, cells were washed twice to remove serum and then incubated in 300 μL/well DMEM with insulin, transferrin and selenium supplements (Sigma, UK) added. Test compounds ((*R*,*R*)-**2b**, (*R*)-**1**) at 0, 1, 5 and 10 μM were added to duplicate wells. After a further 24 h, media were removed for zymographic analysis and the cells were washed in PBS. The cells from each well were harvested by trypsinization, resuspended in PBS and mixed 1:1 with trypan blue (0.4% w/v, Sigma, UK). After 5 min, a sample of cell suspension from each well was removed and introduced into a haemocytometer. The total number of cells in the upper grid was counted and a separate count was taken of the cells, which had taken up the blue dye, that is, the dead cells. The process was repeated by counting the cells in the lower grid. Percentage toxicity for each compound at the different concentrations was calculated as a mean of the values for duplicate wells, after subtracting the values for the wells without either compound.